AD_____

GRANT NUMBER: DAMD17-94-J-4458

TITLE: Characterization of Novel Seven Transmembrane Helix, GS-Coupled Receptors and Pou Domain Proteins in Cancer of the Mammary Epithelium

PRINCIPAL INVESTIGATOR: Michael G. Rosenfeld, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, CA 92093-0934

REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19970618 136

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE
Characterization of Novel Seven Transmembrane Helix, GS-Coupled Receptors and Pou Domain Proteins in Cancer of the Mammary Epithelium

5. FUNDING NUMBERS
DAMD17-94-J-4458

6. AUTHOR(S)
Michael G. Rosenfeld, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
La Jolla, CA 92093-0934

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Understanding the molecular basis of breast carcinoma is central to designing rational and effective therapy for this prevalent disease. We have proposed to investigate several regulatory proteins that we hypothesized may underlie at least a subset of human breast carcinomas. In investigating transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, we have discovered a novel protein, expressed in the breast, that acts as a specific repressor of retinoic acid and thyroid hormone receptors. This protein is a 270kDa protein that is postulated to mediate retinoic acid and thyroid hormone receptor actions as a repressor of specific gene expression. We now have additionally identified a breast-specific form of a co-activator and estrogen and the role of the CREB binding protein in nuclear receptor interaction. These molecules are thus candidates for initiating events in breast tumors. The co-repressor and co-activator and co-integrator proteins together are hypothesized to control mammalian cell proliferation, and the maintenance of mature cell phenotypes, and represent intriguing candidates for involvement in initiation of tumor events.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

MM In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

MM In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

MM In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

M[illegible] 9/26/96

PI - Signature Date

TABLE OF CONTENTS

Front Cover

Standard Form 298

Foreword

Table of Contents

Summary of Progress.....1

Introduction.....2

Body.....3

Nuclear Receptors Directly Interact with CBP.....4
CBP Modulates Ligand-Dependent Transcriptional Activation..4
The Nuclear Receptor-Associated p160 Co-Activator Directly Interacts with CBP.....5
Role of CBP in Nuclear Receptor-Mediated Inhibition of AP-1 Activity.....6

Conclusions.....6

Plans for Next Year.....7
Task I
Task II
Task III
Task IV

References.....8

Appendix.....11
Figure Legend
Figures 1-4

5. SUMMARY OF PROGRESS

Breast cancer is a devastating disease, the etiology of which may be best understood by investigating the regulatory events that can maintain or restore normal growth properties. Our laboratory has investigated control of gene transcription and mammalian development, emphasizing two classes of transcription factors: POU domain factors, and nuclear receptors that mediate both positive and negative patterns of gene expression. Our ongoing studies under this Award are based on the premise that these factors, and trophic receptors under their control, are of specific significance to the etiology of breast cancer.

Our original goals emphasized transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, and a novel seven-transmembrane helix receptor expressed in breast. In the first year of the grant, we continued our investigation of all three problems, but our most important finding was that the discovery of a novel nuclear receptor co-repressor protein, as we described in our progress report in Year 1. We have continued to emphasize this area because it has important implications for a large number of women with breast cancer -- women who have the estrogen receptor positive tumors. In fact, a major problem is the occurrence of resistance to estrogen antagonist widely used in therapy tamoxifen or 4-hydroxy tamoxifen. To begin to understand this, we have studied activation by nuclear receptors which has led to the discovery of important and novel co-activating factors; that is, proteins that are absolutely required for nuclear receptors to activate target genes. **We believe that this coming year we can understand precisely why tamoxifen works as a repressor in breast tissue and breast tumors and the molecular basis of the development of resistance. The nuclear receptor co-repressor (N-CoR) can bind to the estrogen receptor in the presence of the antagonist (partial agonist) tamoxifen. In turn, this is dominant to the actions of co-factors.** We believe that these studies will permit categorization of tamoxifen resistance and new approaches to therapy. **Therefore, we have had a successful year, building on the progress of last year to achieve exciting goals this year. The Army Breast Cancer Grant provides the only source of funding for this research, and we believe that we are in a position to complete the drug resistant aspects of estrogen receptor positive tumors under the support of this grant.**

The POU domain factor we found to be expressed in breast was found to also be expressed in several other tissues, and we have proceeded to construct a knockout vector which has been electroporated into embryonic stem cells, and homologous recombination has been observed. These ES cells will be injected to attempt to achieve germ line transmission and by proper breeding obtain amounts null for their genomic locus. Effects on breast development will be evaluated. The seven transmembrane helix gene has also been investigated by gene knockout approach as originally proposed; at this point we have obtained chimeric mice which are being bred to obtain germ line transmission. **Therefore, over the course of the year, these two studies will continue to proceed as originally proposed; however, our major emphasis is clearly focused on the estrogen receptor project because, as one should do in scientific research, we have chosen the most interesting project on which to focus our efforts under this grant, believing this will be to the maximum beneficial impact.**

Nuclear receptors are clearly important in the biology of breast tumors, the retinoic acid receptor appears to exert critical roles in normally preventing abnormal proliferation events. Altered control by defects in the regulation of the retinoic acid receptor is likely to serve in initiation events in breast cancer. In turn, retinoic acid receptor appears to require a co-expression to mediate its normal control of growth. Under this grant, we identified a novel protein that appears to be the long-sought retinoic acid receptor co-repressor, the function of which is regulated by allosteric

effects imparted by the DNA sites to which retinoic acid receptor is bound. On specific DNA sites, this putative co-repressor protein associates with, and is released by binding of ligand, while on other sites it is not released by binding of ligand. This novel molecule has clear implications with respect to initiation of abnormal growth and loss of differentiation in mammary epithelium. Based on the nature of the specific DNA sites to which retinoic acid receptor is bound, this factor is postulated to be a central aspect of the ability of nuclear receptors to promote growth in some cell types, and inhibition of growth in others, we hypothesize that environmental conditions and genetic predisposition alter regulation of this factor.

In the past few months, we have discovered that, unlike estrogen receptor or estradiol based estrogen receptor, which N-CoR does not bind, that tamoxifen used for estrogen-receptor-positive in breast cancers causes N-CoR to bind estrogen receptor. This in turn prevents co-activators from activating the receptor. Tamoxifen resistance in breast tumors is thus likely to reflect alterations in the co-repressor complex activity.

INTRODUCTION

The morphogen retinoic acid is required for development, growth and differentiation (reviewed in 1 and 2). Retinoids, a group of analogs of vitamin A, particularly at high levels, suppress carcinogenesis in various epithelial tissues, including the mammary gland (3-6). We believe that this reflects the actions with a co-repressor that we believe may serve roles in initiation of breast cancer. The hormone effects are mediated by binding to specific nuclear receptors (3) that are members of the steroid/thyroid hormone receptor superfamily (4-6). This class of proteins function as ligand dependent transcription factors that mediate the response of the hormone signal by direct control of gene expression. The estrogen and glucocorticoid receptors bind DNA as homodimers, while retinoic acid receptors preferably interact with their cognate DNA response elements as components of heterodimeric complexes (7-20) often involving a partner that our laboratory and others identified to be members of the retinoid X receptor (RXR) family (8-21). Heterodimers of retinoic acid receptor and retinoid X receptor bind with high affinity and activate transcription from response elements consisting of direct repeats, palindromic, or inverted palindromic arrangements of a core recognition motif (4, 8-21). The relative orientation and spacing of the core recognition motifs play essential roles in the specificity of DNA binding and transcriptional activation. While heterodimers of retinoic acid receptor and retinoid X receptor bind to direct repeats of core motifs spaced by 1, 2 and 5 bp (DR+1, DR+2, and DR+5), an unspaced palindrome binds to an inverted palindromic arrangement of the core motif spaced by 6-8 bp, (e.g. IP+6) (reviewed in 22, 23).

Recent studies indicate that heterodimeric complexes of retinoic acid receptor and retinoid X receptor molecules exhibit a polarity binding to various DNA elements (24-26), and that this polarity-specific binding may play important roles in cell-specific regulation by retinoic acid receptors. On a direct repeat spaced by 5 bp (DR+5) retinoid X receptor selectively binds to the upstream half-site and retinoic acid receptor binds to the downstream half-site (25,26). When the spacing is reduced by one basepair (DR+4 site) the element becomes a binding site for heterodimers of thyroid hormone receptor and retinoid X receptor. In this case the thyroid hormone receptor is bound to the downstream half-site and the retinoid X receptor again interacts with the upstream half-site. However, in the case of a direct repeat spaced by 1 bp (DR+1) retinoid X receptor binds to the downstream half-site and retinoic acid receptor binds to the upstream half-site (27). The stringency of this polarity-specific binding was further confirmed using specific mutants of retinoid X receptor containing the P-box residues of the glucocorticoid receptor (27). In contrast to the glucocorticoid receptor, retinoic acid receptors are not associated with heat shock proteins in the absence of hormone, but are bound to their response elements and are able to actively repress basic transcription (28,29).

Our laboratory and others, were able to show that this repression is mediated by the C-

termini of the retinoic acid and thyroid hormone receptors (30-33). Thus, thyroid hormone and retinoic acid receptors bind as heterodimers with the retinoid X receptor in a polarity-specific fashion to cognate DNA sites that are generally organized as direct repeats of a core binding motif (17, 36-44), and as a consequence of binding to these sites can exert either positive control of gene transcription or repress gene transcription.

The cellular thyroid hormone receptor, like the viral oncogene erbA of the Avain Erythroblastosis Virus (AEV), V-erbA, represses transcription of target genes in the absence of ligand, with hormone binding resulting in de-repression and activation (31,34,35,45). Evidence has indicated that in most cases ligand-independent repression appears to result from an active repressor functions within the ligand binding domain. A ligand-independent repression function could be transferred by the carboxyl-terminal region of the thyroid hormone receptor to heterologous DNA binding domain. Fusion of the C-terminal domains of v-erbA. T_3R, and RAR to the DNA binding domain of the yeast transcription factor GAL4, generated UAS-dependent transcriptional repressor proteins (31). In contrast, the RXR C-terminus fused to the GAL4 DNA binding domain did not mediate transcriptional silencing.

The molecular mechanism responsible for nuclear receptor transcriptional silencing are not well understood. Recent studies showed that several nuclear receptors may interact with the basal transcription factors, including TFIIβ (47); however, the distal T_3 receptor thyroid hormone C-terminal regions that interact with TFIIβ, are not sufficient to confer repression. Indeed, the regions in the hinge and N-terminal part of the ligand-binding domain of the thyroid hormone receptor are required for silencing (46,47). Co-transfection experiments suggest that these sequences which do not bind TFIIβ can potentially compete for a putative soluble co-repressor molecule (47) and imply the existence of additional interacting factors that are required for ligand-independent repression. Last year, under this Grant, we isolated and characterized a novel 270 kDa factor (N-CoR) characterized by an interaction domain in the distal C-terminus, and a transferable repressor domain in the N-terminus. This factor interacts with the thyroid hormone receptor in the mutant cell and also interacts with retinoid acid receptor, but not with the unliganded estrogen, progesterone, retinoid X, glucocorticoid, or vitamin D receptors. Receptor specificity is explained by specific regions in the hinge region and the adjacent ligand-binding domain that is present in all thyroid hormone and retinoic acid receptors, as well as in v-erbA. Because specific mutations in this region that abolished interactions with the 270 kDa protein also eliminated the ligand-independent repression function of the thyroid hormone receptor, retinoic acid, and because 270 kDa protein can itself function as a repressor, our data suggested that the 270 kDa protein associated with the unliganded, DNA-bound thyroid hormone receptor, and retinoic acid is required for ligand-independent transcriptional repression; we, therefore, termed this protein N-CoR for nuclear receptor co-repressor (51-53).

Several lines of evidence indicate that nuclear receptors must interact with additional factors dependent on a conserved distal C-terminal motif (AF2) to mediate both activation and repression of gene expression (48-52). Biochemical assays have identified 140 and 160 kDa proteins (p140 and p160) (49,48,52) that associate with estrogen, retinoic acid, thyroid hormone, retinoid X, and potentially other nuclear receptors as the most prominent ligand-dependent putative co-activators, binding in an AF2-dependent fashion. In addition, a series of proteins exhibiting ligand-dependent interactions with the C-termini of nuclear receptors that may also function as co-activators have been identified using a yeast two-hybrid screen.

6. BODY

Over the past year, we have worked to identify the p160 exhibiting ligand-dependent association with DNA-bound thyroid hormone and retinoic acid receptors in the presence of thyroid hormone. The ligand-binding domains of estrogen receptor and other nuclear receptors,

including retinoic acid and thyroid hormone receptors, interact strongly in the cell with a conserved domain in the N-terminus of CBP and p300 in a ligand-dependent manner. Further, the putative co-activator p160 was found to interact independently and specifically with a conserved C-terminal domain in CBP and p300. Expression cloning of a family of p160 cDNAs was achieved based on estrogen receptor and CBP interaction. Several independent experimental approaches have suggested a central role for CBP in ligand-dependent activation of RAR and T3R, comparable to its role for CREB and Jun/Fos. Our data indicate competition for limiting amounts of CBP may account for many of the inhibitory actions of both GR and RAR on AP-1 activation. We propose that CBP/p300 is a required component common to the co-activator complexes of diverse transcription factors, each utilizing specific additional co-activators, thus providing a mechanism for integrating in the nucleus diverse signaling pathways in the maintenance of homeostasis. One of the p160 factors (NCoA-1) is associated directly with liganded estrogen receptors, while a second (p/CIP) is associated with CBP; both are required for estrogen-dependent activation.

Nuclear Receptors Directly Interact with CBP

To investigate possible direct interactions between estrogen receptors and CBP, a series of overlapping fragments of the 265 kDa CBP protein were bacterially expressed as glutathione-S-transferase (GST) fusion proteins, and tested for interaction with retinoic acid receptor, revealing a strong ligand-dependent interaction confined to a single N-terminal CBP region. The ability of this interaction to occur in intact cells in a ligand-dependent manner was confirmed using the yeast two-hybrid system. Further mapping revealed that the N-terminal 100 amino acids of CBP retained full interaction with estrogen and retinoic acid receptors; attempts to further subdivide this region resulted in a complete loss of capacity to interact.

Consistent with the ability of several nuclear receptors to inhibit the activated AP-1 transcriptional response, we found that estrogen, thyroid hormone, and retinoid X receptors, also exhibited strong ligand-dependent interactions, with the identical N-terminal domain of CBP, dependent upon a functional AF2 domain. Intriguingly, a point mutation outside of the AF2 domain that conferred dominant-negative functions to the RAR (G303-E) also abolished interactions with CBP. Together these data indicate that CBP binds to the C-terminal domain of ligand-occupied nuclear receptors. These interactions were demonstrated to occur on DNA, using the protein-dependent gel mobility shift assay, in which addition of an N-terminal fragment of CBP (amino acids 1-450) caused a marked supershift of the RAR/RXR, DNA complex, only in the presence of ligand. The specificity of this interaction was established because the region of CBP interacting with phosphorylated CREB (amino acids 450-720) failed to supershift RAR/RXR, DNA complexes (Figure 1).

Further, assays established that ligand-dependent interactions between retinoic acid receptor and CBP occurred *in vivo*. Further, specific *in vivo* interactions with glucocorticoid and retinoid X receptors were demonstrated.

CBP Modulates Ligand-Dependent Transcriptional Activation

Based on the *in vivo* interaction between the nuclear receptors and CBP, it became of interest to evaluate the potential role of these interactions in ligand-dependent functions of nuclear receptors. Although CBP is widely expressed, its low levels are rate-limiting for CREB-mediated transcription, which permitted co-transfection assays to evaluate a role for CBP in CREB-dependent transcription. We, therefore, examined ligand-dependent transcription from a retinoic acid response element containing promoter in the presence and absence of co-expressed CBP. Expression of CBP led to a three-fold further enhancement of retinoic acid-dependent transcription, but had little or no effect on promoters lacking a RARE (Figure 2). To acquire further, independent evidence for a potential role of CBP in RAR-mediated transcription, in vitro

transcription assays were performed using nuclear extracts prepared from cells with varied levels of CBP (Figure 2). To be certain that the effects of CBP were exerted through the ligand-binding domain of nuclear receptors, we utilized a GAL4-T_3 receptor fusion protein. The ability of this fusion protein to activate the TK promoter containing GAL4 binding elements (17 mer UAS), was assessed using GAL4 protein as a control. T_3-induced transcription of this UAS-driven reporter was increased >5-fold by additional CBP, while altered levels of CBP had little effect on transcription of several control promoters. Increased levels of CBP also markedly increased AP-1 site-dependent reporter expression, consistent with the proposed role of CBP in AP-1 transactivation events, and indicating that CBP is limiting in extracts used in *in vitro* transcription assays.

Based on these suggestive results, the role of CBP, or highly related factors, in ligand-dependent transcriptional activation was directly tested by evaluating the ability of microinjected anti-CBP IgG to inhibit ligand-dependent transcription from RARE-containing promoters in intact cells. In each experiment, >200 cells were injected with lacZ reporter plasmids and either control or anti-CBP IgG; cells were treated with all-trans retinoic acid, stained with X-gal, and positive cells were counted. In these assays, addition of a ligand was required for effective expression of two independent RARE-dependent reporters, but did not affect other promoters not containing RAREs. In three independent experiments, co-injection of increasing concentrations of anti-CBP IgG, but not control IgG, resulted in a progressive and specific decrease in ligand-dependent gene activation, producing >80% inhibition of ligand-induced activation. Even in the remaining 20% of cells that scored positive, there was often a reduced signal compared to levels in cells injected with control IgG. These data revealed that, in a concentration-dependent fashion, anti-CBP IgG specifically inhibited ligand-dependent activation of transcription units containing retinoic acid response elements, without altering expression of other promoters.

The Nuclear Receptor-Associated p160 Co-Activator Directly Interacts with CBP

Because CBP and/or its related family members were required for transactivation by retinoic acid and other nuclear receptors, we investigated the possibility that putative nuclear receptor co-activators, p140 or p160, could themselves interact with CBP. A series of GST-fusion proteins spanning the entire sequence of CBP was used in interaction assays with extracts from various cell types. Specific interactions were identified between the C-terminal region of CBP and p160, but not with p140, distinct from the internal regions of CBP that interact with CREB, Fos, TFIIß, and E1A. To better define the determinants of p160 interaction, a series of fragments were generated from the CBP C-terminus, revealing a region of 105 amino acids that was sufficient for interactions with p160. In addition, ^{32}P-CBP C-terminus could detect p160 in Far-Western experiments.

cDNAs encoding the putative p160 (nuclear receptor co-activator, NCoA) were obtained by expression cloning based on the criteria that phage plaques exhibited interaction with both the CBP C-terminus and liganded nuclear receptors. These gene products were identified (Figure 3). The first (NCoA-1) was represented as a series of cDNAs were identified that encoded variant forms of the SRC-1 protein reported to have a predicted molecular weight of 115 kDa (54). The N-terminally extended variants that we identified included forms of 1465 and 1405 amino acids, with predicted molecular weights of 159 and 152 kDa. We also identified two additional isoforms (referred to as NCoA-2, NCoA-3), one of which is selectively expressed in the breast. The N-terminal sequence of each protein exhibited a homology with the PAS domain characteristic of the PAS, HLH gene family. These related factors exhibited ~40% identity in their CBP and nuclear receptor interaction domains. Anti-serum raised against an internal 50 kDa region common to each protein identified recombinant NCoA-1 proteins as the 160 kDa protein doublet or triplet in whole cell extracts. Based on immunodepletion experiments, these p160 factors are the biochemically-identified p160. Microinjection assays confirmed that NCoA-1 and NCoA-3 were required for

estrogen and retinoic acid receptor-dependent activation events. Nuclear receptors and p160 interact with conserved motifs present in CBP and the p300, representing a coiled-coil interaction domain.

Role of CBP in Nuclear Receptor-Mediated Inhibition of AP-1 Activity

In parallel, we wished to explore a potential role for CBP in mediation of ligand-dependent inhibition of AP-1 function. To begin to test this hypothesis, we established a transfection assay in which liganded retinoic acid or glucocorticoid receptors could effectively inhibit (~80%) phorbol ester-mediated activation of AP-1-dependent promoters. If competition for limiting quantities of CBP accounts for the inhibitory effect of nuclear receptors, then increased levels of CBP should restore AP-1-dependent activation. Indeed, the inhibitory effect of liganded retinoic acid and glucocorticoid receptors were largely or completely abolished by co-transfection of vectors expressing CBP. Co-transfection with vectors expressing p300 also diminished the AP-1 inhibitory effects of nuclear receptors, but appeared somewhat less effective than CBP. Mutations in the retinoic acid receptor AF2 domain that inhibit binding of CBP and other co-activator proteins abolished AP-1 repression by nuclear receptors, consistent with previous data involving C-terminal receptor deletions. The CBP-dependent inhibition of AP-1 activity could formally reflect either an allosteric effect whereby binding of nuclear receptors could preclude binding of the AP-1 complex to CBP or a competition for limiting amounts of CBP. However, the first possibility appears unlikely because we found that binding of nuclear receptors (RAR) and Jun could simultaneously occur on CBP.

A recently described synthetic retinoic acid receptor ligand (LG550) exerts a potent anti-AP-1 effect, while LG629 serves as an antagonist. Based on the model that CBP partitioning can mediate trans-repression of AP-1 by nuclear receptors, it would be predicted that LG550 might result in potent association with CBP. This possibility was initially assessed using GST-RAR fusion proteins, which revealed significantly enhanced binding of CBP N-terminus in the presence of LG550, compared to all-trans retinoic acid; while interaction was abolished by the antagonist, LG629. Further, using the yeast two-hybrid assay, LG550 was found to be a more potent stimulator of CBP/retinoic acid receptor interactions than all-trans retinoic acid, while LG629 abolished interaction. Finally, interactions in intact cells were assessed by immunoprecipitation of CBP-containing complexes after addition of various ligands. CBP/retinoic acid receptor interactions were markedly increased when LG550 was added, compared to cells treated with all-trans retinoic acid, and the interaction was abolished by LG629. Together, these observations are consistent with the hypothesis that the interaction between retinoic acid receptor and CBP is responsible for some forms of repression of AP-1 activity. If this were the case, one would predict that CREB, which appears to have a very high affinity for CBP, might be able to actually inhibit nuclear receptor activation. This possibility was tested by transfection of an RARE-dependent reporter and CMV-CREB and retinoic acid-dependent induction, after treatment of cells with 8-Br-cAMP. Indeed, activated CREB reduced retinoic acid-dependent stimulation from >40-fold to ~5 fold, without affecting the basal activity of the RARE-driven reporter.

7. CONCLUSIONS

We propose that the CBP/NCoA-1 co-activator complexes together serve as "integrators," based on their role in determining the relative transcriptional responses of a specific target gene in the face of activation of multiple signalling pathways. The relative abilities of co-activators to recruit limiting amounts of CBP into integrator complexes could account for aspects of the multifactorial control of biological processes under regulation of multiple signal transduction pathways. Thus, the precise cohort of genes activated in a given cell will depend both on the relative activity of diverse signalling pathways, and the organization and relative affinity of the complex on various DNA-bound factors that utilize CBP as a co-activator. Synergy might be

expected on some promoters, while competitive interaction would be expected for others that contain only a subset of sites for interacting transcription factors. Thereby, the CBP/p300 family serves to integrate the signals of diverse growth factors, hormones and intracellular ligands that combinatorially modulate homeostasis and proliferation.

The discovery of a second, required p160 co-activator of the estrogen and retinoic acid receptors led to the discovery of a related co-activator (NCoA-3) that has distinct functions and interacts with CBP, rather than the nuclear receptor. We have now discovered that clinically used estrogen antagonists (tamoxifen) causes NCoA-3 to bind to estrogen receptor, preventing its functions in gene activation. Finally, we have preliminary data that tamoxifen causes NCoR to bind to estrogen receptor recruiting a co-repressor complex, which includes a deacetylase (Figure 4). We can now determine whether this step becomes defective in development of tamoxifen resistance.

PLANS FOR NEXT YEAR

As outlined in our Plans in last year's Progress Report, this year we plan:

Task I To generate animals null for the BrR receptor factor genomic loci. Injection of ES cells with constraints are being performed.

Task II (Continuation from Year 1 Progress Report) Further investigate the nuclear receptor co-repressor, N-CoR, and prove it is a co-repressor *in vivo.*

Task III (Continuation from Year 1 Progress Report) Study the recently identified novel breast estrogen receptor co-activator.

Task IV (Initiated from Year 2) Link the co-repressor and co-activator to drug-resistant breast cancer. We will test the model that inhibitors (tamoxifen) used in clinical breast cancer cause estrogen receptor to bind the nuclear receptor co-repressor, and failure of this association causes tamoxifen resistance.

8. REFERENCES

1. Schwartz, H.L. (1983). Molecular Basis of Thyroid Hormone Action. Academic Press pp.413-444.
2. Ragsdale, C.W. and Brockes, J.P. (1991). Nuclear Hormone Receptors. Academic Press pp.235-259.
3. Bigler, J. and Eisenmann, R.N. (1988). C-erbA encodes multiple proteins in chicken erythroid cells. Mol.Cell.Bol. 8, 4155-4161.
4. Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.
5. Green, S. and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. 4, 309-314.
6. Beato, M. (1989) Gene regulation by steroid hormones. Cell 56, 335-344.
7. Glass, C.K., Lipkin, S.M., Devary, O.V., and Rosenfeld, M.G. (1989) Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. Cell 59, 697-708.
8. Forman, B.M., Yang, C.R., Au, M., Casanova, J., Ghysadael, J., and Samuels, H.H. (1989) A domain containing leucine-zipper-like motifs mediate novel in vivo interactions between the thyroid hormone and retinoic acid receptors. Mol.Endo. 3, 1610-1626.
9. Murray, M.B. and Towle, H.C. (1989) Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol.Endo. 3, 1434-1442.
10. Burnside, J., Darling, D.S., and Chin, W.W. (1990) A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. J.Biol.Chem. 265, 2500-2504.
11. Rosen, E.D., O'Donnell, A.L., and Koenig, R.J.(1992) Ligand-dependent synergy of thyroid hormone and retinoid X receptors. J.Biol.Chem. 267, 22010-22013.
12. Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., and Evans, R.M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358, 771-774.
13. Leid, M., Kastner, P., and Chambon, P. (1992) Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem.Sci. 17, 427-433.
14. Marks, M.S., Hallenbeck, P.L., Nagata, T., Segars, J.H., Appella, E., Nikodem, V.M., and Ozato, K. (1992) H-2RIIBP (RXR beta) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. 11, 1419-1435.
15. Zhang, X.K., Lehmann, J., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature 358, 587-591.
16. Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991) A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell 66, 555-561.
17. Umesono, K., Murakami, K.K., Thompson, C.C., and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell 65, 1255-1266.
18. Näär, A.M., Boutin, J.M., Lipkin, S.M., Yu, V.C., Holloway, J.M., Glass, C.K., and Rosenfeld, M.G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 65, 1267-1279.
19. Katz, R.W. and Koenig, R.J. (1993). Nonbiased identification of DNA sequences that bind thyroid hormone receptor alpha 1 with high affinity. J.Biol.Chem. 268, 19392-19397.
20. Luisi, B.F., Xu, W.X., Otwinowski, Z., Yamamoto, K.R., and Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA.

Nature 352, 497-505.
21. Umesono, K., Giguere, V., Glass, C.K., Rosenfeld, M.G. and Evans, R.M. (1988). Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature 336, 262-265.
22. Yu, V.C., Näär, A.M., and Rosenfeld, M.G. (1992) Curr.Opinion Biotech. 3, 597-602.
23. Glass, C.K. Endocrine Reviews. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. 15, 391-407.
24. Yen, P.M., Ikeda, M., Brubaker, J.H., Forgione, M., Sugawara, A., and Chin, W.W. (1994) Roles of v-erbA homodimers and heterodimers in mediating dominant negative activity by v-erbA. J.Biol.Chem. 269, 903-909.
25. Kurokawa, R., Yu, V.C., Näär, A., Kyakumoto, S., Han, Z., Silverman, S., Rosenfeld, M.G., and Glass C.K. (1993) Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. Genes & Dev. 7, 1423-1435.
26. Predke, P.F., Zamble, D., Sarkar, B., and Giguere, V. (1994) Ordered binding of retinoic acid and retinoid-X receptors to asymmetric response elements involves determinants adjacent to the DNA-binding domain. Mol.Endo. 8, 31-39.
27. Kurokawa, R., DiRenzo, J., Boehm, M.F., Sugarman, J., Glass, B., Rosenfeld, M.G., Heyman, R.A., and Glass, C.K. (1994) Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. Nature 371, 528-31.
28. Levine, M. and Manley, J.L. (1989) Transcriptional repression of eukaryotic promoters. Cell 59, 405-408.
29. Renkawitz, R. (1990) Transcriptional repression in eukaryotes. Trends Genet. 6, 192-197.
30. Holloway, J.M., Glass, C.K., Adler, S., Nelson, C.A., and Rosenfeld, M.G. (1990) The C'-terminal interaction domain of the thyroid hormone reion factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. J.Biol.Chem. 268, 4152-4154.
31. Baniahmad, A., Tsai, S.Y., O'Malley, B.W., and Tsai, M.J. (1992) Kindred S thyroid hormone receptor is an active and constitutive silencer and a repressor for thyroid hormone and retinoic acid responses. Proc.Natl. Acad.Sci.USA 89, 10633-10637.
32. Fondell, J.D., Roy, A.L., and Roeder, R.G. (1993) Unliganded thyroid hormone receptor inhibits formation of a fuion factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. J.Biol.Chem. 268, 4152-4160.
33. Chambon, P. (1995) The molecular and genetic dissection of the retinoid signaling pathway. Recent. Prog. Horm. Res. 50, 317-332.
34. Sap, J., Muñoz, A., Schmitt, J., Stunnenberg, H. and Vennstrom, B. (1989) Repression of transcription mediated at a thyroid hormone response element by the v-erb-A oncogene product. Nature 340, 242-244.
35. Damm, K., Thompson, C.C., and Evans, R.M. (1989) Protein encoded by v-erb-A functions as a thyroid-hormone antagonist. Nature 339:593-597.
36. Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Naar, A.M., Kim, S.Y., Boutin, J. M., Glass, C.K. and Rosenfeld, M.G. (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67, 1251-1266.
37. Kliewer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature 355, 446-449.
38. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders M., Zacharewski, T., Chen, J.Y., Staub, A., Garnier, J.M., Mader, S. and Chambon, P. (1992) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68, 377-395.
39. Marks, M.S., Hallenbeck, P.L., Nagata, T., Segars, J.H., Appella, E., Nikodem, V.M. and Ozato, K. (1992) H-2RIIBP (RXR beta) heterodimerization provides a mechanism

for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. 11, 1419-1435.

40. Zhang, X.-K, Hoffmann, B., Tran, P.B.-V., Graupner, G. and Pfahl, M. (1992) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature 355, 441-446.
41. Kurokawa, R., Yu, V., Näär, A., Kyakumoto, S., Han, Z., Silverman, S., Rosenfeld, M.G. and Glass, C.K. (1993) Differential orientations of the DNA binding domain and C-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. Genes & Dev. 7, 1423-1435.
42. Perlmann, T., Rangarajan, P.N., Umesono, K. & Evans, R.M. (1993) Determinants for selective RAR and TR recognition of direct repeat HREs. Genes & Dev. 7, 1411-1422.
43. Näär, A.M., Boutin, J.M., Lipkin, S.M., Yu, V.C., Holloway, J.M., Glass, C.K. and Rosenfeld, M.G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 65, 1267-1279.
44. Glass, C.K., Devary, O.V. and Rosenfeld, M.G. (1990) Multiple cell type-specific proteins differentially regulate target sequence recognition by the alpha retinoic acid receptor. Cell 63, 729-738.
45. Muñoz, A., Zenke, M., Ulrich, Gehring, Sap, J., Beug, H. and Vennström, B. (1988) Characterization of the hormone-binding domain of the chicken c-erbA/thyroid hormone receptor protein. EMBO J. 7, 155-159.
46. Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M-J. and O'Malley, B.W. (1993) Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormones. Proc. Natl. Acad. Sci. USA 90, 8832-8836.
47. Baniahmad, A., Lendg, X., Burris, T.P., Tsai., S.Y., Tsai, M. and O'Malley, B.W. (1995) The TAU4 activation domain of the thyroid hormone receptor is required for release of a putative co-repressor(s) necessary for transcriptional silencing. Mol.Cell.Biol. 15, 176-186.
48. Cavailles, V., Dauvois, S., Danielian, P.S., and Parker, M.G. (1994). Interaction of proteins with transcriptionally active estrogen receptors. Proc. Natl. Acad. Sci. USA 91, 10009-10013.
49. Halachmi, S., Marden, E., Martin, G., MacKay, I., Abbondanza, C., and Brown, M. (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 264, 1455-1458.
50. Baniahmad, A., Leng, X., Burris, T.P., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). The tau4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol. & Cell. Biol. 15, 76-86.
51. Hörlein, A.J., Näär, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., Glass, C.K., and Rosenfeld, M.G. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377, 397-404.
52. Chen, J.D., and Evans, R.M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454-457.
53. Kurokawa, R., Söderström, M., Hörlein, A., Halachmi, S., Brown, M., Rosenfeld, M.G. and Glass, C.K. (1995). Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature 377, 451-454.
54. Oñate, S.A., Tsai, S.Y., Tsai, M.-J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270, 1354-1357.

APPENDIX

Figure 1: Ligand-dependent interactions between CBP and nuclear receptors *in vivo* and *in vitro*.

A. Specific regions of CBP interact with liganded retinoic acid and estrogen receptor C-terminal domains. A series of GST-CBP fusion proteins, encompassing the entire CBP protein or GST alone, were subjected to SDS-PAGE, transferred to nitrocellulose and tested for their ability to interact with ^{32}P-labelled estrogen receptor (ER) C-terminus in the absence or presence of 10^{-6} M β-estradiol. Equal amounts of protein were loaded. The arrow indicates the CBP fragment that interacts with ER. **B**. The yeast two-hybrid system reveals that CBP contains a domain that specifically interacts with retinoic acid receptor in the presence of ligand (10^{-7}M TTNPB). **C**. Several members of the nuclear receptor gene family interact with CBP in the yeast two-hybrid system. CBP N terminus (amino acids 1-450) was used as a prey. Interaction of LexA fusion proteins encompassing the C-terminal ligand binding domains of T_3R, RXR, RAR, and ER were measured in the presence or absence of the appropriate ligands. T_3R E403–>Q contains a mutation in the AF2 region. RAR Δ403 has a C-terminal deletion at amino acid 403 of RARα. RAR G303–>E is a dominant-negative mutation, and RXR E454–>Q contains a mutation in the AF2 region, which renders it incapable of gene activation. β-Galactosidase activity was determined as described above. Ligands used were TRIAC (10^{-7}M) for T_3R, TTNPB (10^{-7}M) for RAR, LG69 (10^{-7}M) for RXR, and β-estradiol (10^{-7}M) for ER.

Figure 2: Role of CBP in RAR and T_3R gene activation events.

A. Co-transfection of CMV-CBP plasmids in HeLa cells enhances retinoid acid receptor-mediated transactivation. **B**. Increased CBP levels potentiate hormone dependent transactivation by a GAL4-T_3R C-terminus fusion protein. *In vitro* transcription assays were performed with luciferase reporters driven by 3x UAS(GAL)-tk, human papilloma virus (HPV-16) or a TPA response element upstream of the prolactin minimal promoter (Drolet et al., 1991) (TRE-p36). **C**. Microinjection of anti-CBP antibody abrogates ligand-dependent gene activation by the retinoic acid receptor. Plasmids consisting of a lacZ reporter under the transcriptional control of retinoic acid response elements were injected into the nuclei of Rat-1 cells. RARE SV40 lacZ contains 3 copies of the DR+5 element upstream of the SV 40 minimal promoter, whereas the RARE p36 lacZ contains 2 copies of DR+5 upstream of the prolactin minimal promoter. Each construct was injected with preimmune IgG or with affinity purified anti-CBP antibody and demonstrated a retinoic acid dependent response. The expression of the reporter plasmids was monitored by Xgal staining and quantitated based on the percentage of injected cells which stained blue. Co-injection of the same anti-CBP antibody did not inhibit expression of the reporters driven by either the SV40 or CMV viral enhancers.

Figure 3: The p160 gene family: interactions with CBP and liganded nuclear receptors.

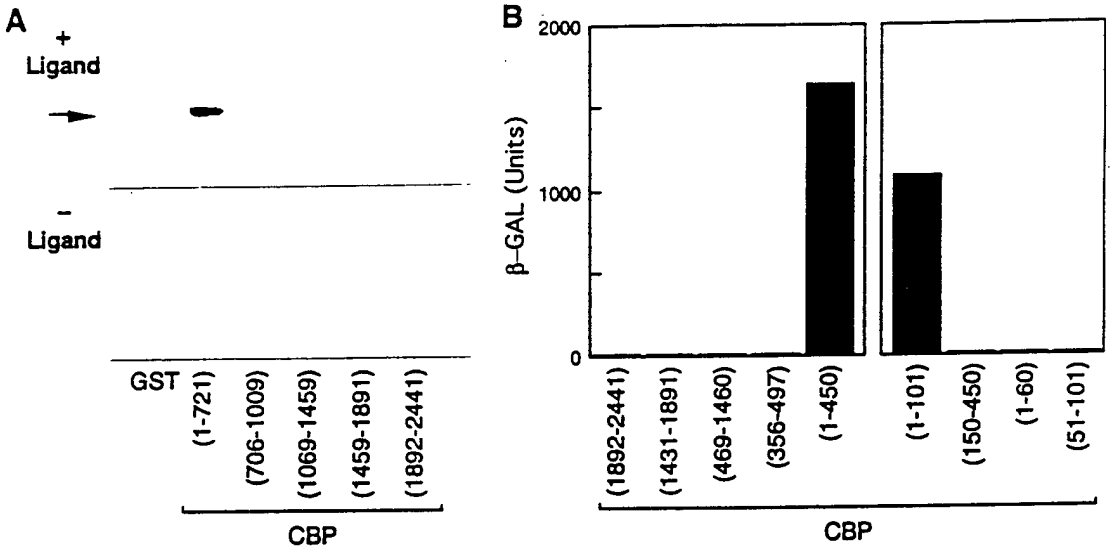
A. A mouse pituitary and a human macrophage λgt11 cDNA library (Lin et al., 1992) were screened for proteins interacting with ^{32}P-labelled ligand-bound estrogen receptor C-terminus. Eleven positive plaques were identified from 1.5 x 10^6 plaques evaluated, seven of which also interacted with the ^{32}P-CBP-GST-C-terminus (amino acids 2058-2163). **B.** p160 is antigenically related to SRC-1. A guinea pig polyclonal antiserum was raised against a GST fusion of a 500 amino acid region common to all SRC-1 variants, encompassing the nuclear receptor and CBP interaction domains. This antiserum was used to immunodeplete reactive proteins from HeLa whole cell extracts (Lanes 3 and 7). Pre-immune serum was used as a control (Lanes 4 and 8).

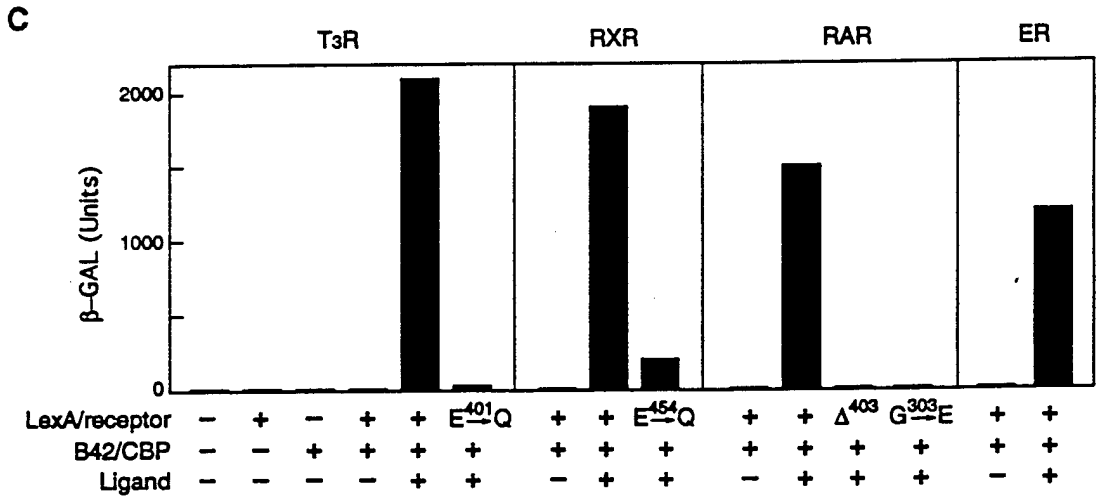
Treated and untreated whole cell extracts were incubated with GST-RAR bound to glutathione-agarose, in the presence or absence of all-trans retinoic acid, to purify putative co-activator proteins. Following extensive washing, specifically bound proteins and GST-RAR were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first probed with liganded ^{32}P-GST-estrogen receptor to detect p140 and p160 (Lanes 2 and 6). Pre-treatment of HeLa extracts with SRC-1 antiserum, but not pre-immune serum, resulted in a marked decrease in the p160 band detected by labelled estrogen receptor (compare lanes 2 and 3). The membrane was then stripped and probed with anti SRC-1 IgG. This demonstrated near quantitative (>75%) removal of the p160 recognized by specific antiserum during the initial immunodepletion (compare Lanes 6 and 7). **C.** Nuclear receptors, CBP and SRC-1 can form a ligand-dependent ternary protein complex. A protein-protein interaction assay was performed with GST-CBP (1-450), which contains only the nuclear receptor interaction domain, bacterially expressed His-T_3R and *in vitro* translated ^{35}S-labelled SRC-1 in the absence or presence of 10-6 M TRIAC (lanes 2 and 3). GST alone was used as a negative control (lane 1) and 25% of SRC-1 input is shown (lane 4). **D.** A mouse cDNA encoding a 158 kDa variant of SRC-1: Sequencing of murine and human cDNA isolates revealed an ORF predicting a protein of 1465 amino acids, containing an N-terminal extension beyond the sequence of human SRC-1 with high homology (~88% identity) over the rest of SRC-1. Additional variants of SRC-1 with distinct N-terminal (SRC-1b) or C-terminal sequences (SRC-1c,d), reflecting alternative splicing events were detected.

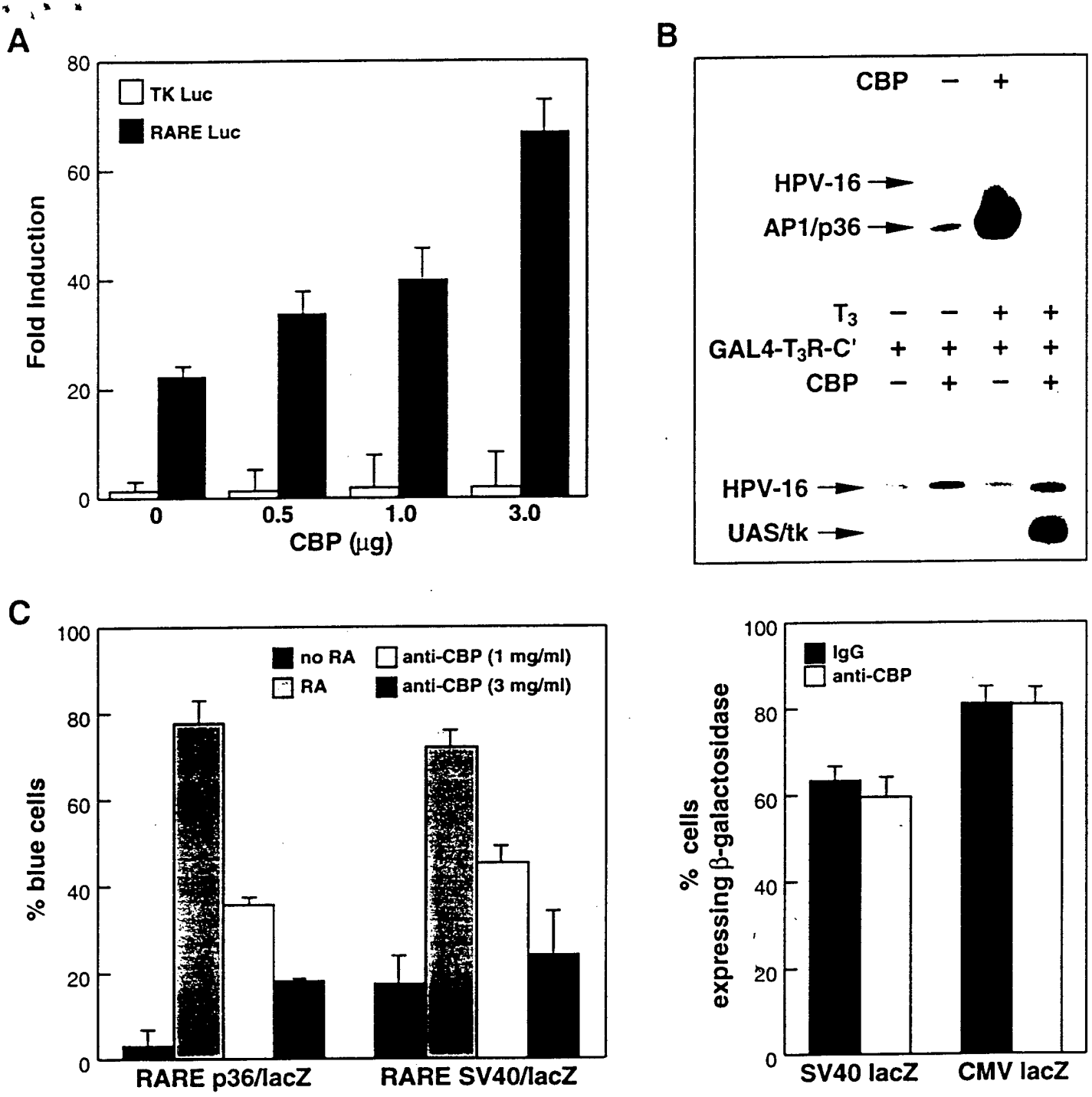
Figure 4: Model of acetylase and estrogen receptor action.

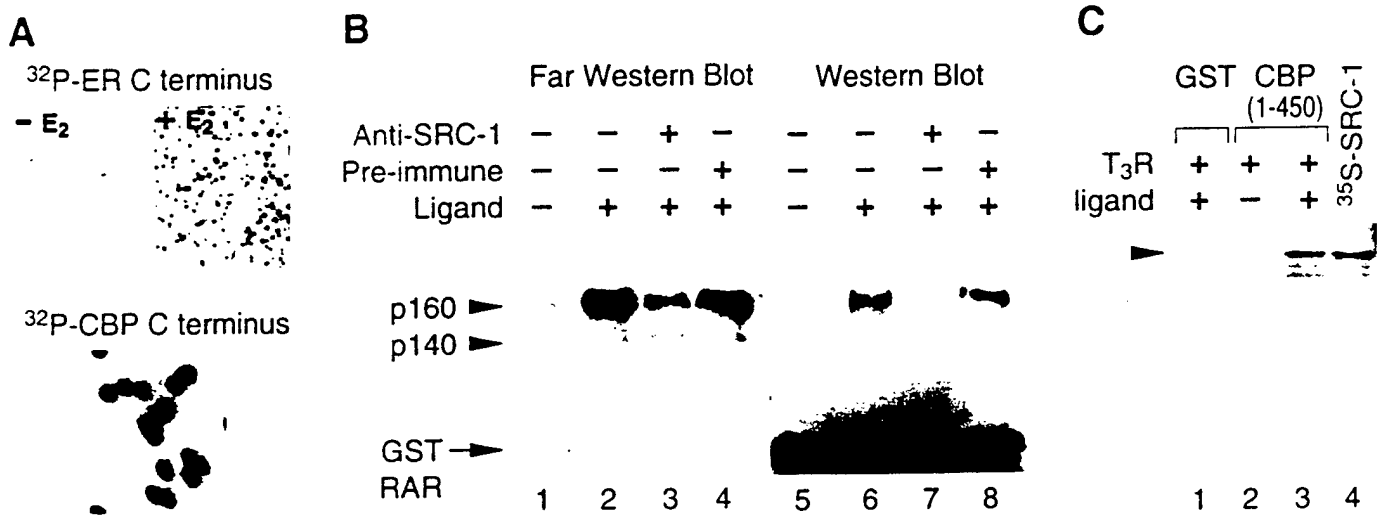
An NCoR-dependent co-repressor complex mediates repression; while a CBP/NCoA complex mediates ligand-dependent gene activation.

Figure 1


A
+
Ligand
-
Ligand
GST
(1-721)
(706-1009)
(1069-1459)
(1459-1891)
(1892-2441)
CBP
B
2000
1000
0
β-GAL (Units)
(1892-2441)
(1431-1891)
(469-1460)
(356-497)
(1-450)
(1-101)
(150-450)
(1-60)
(51-101)
CBP


C
T3R
RXR
RAR
ER
2000
1000
0
β-GAL (Units)
LexA/receptor
B42/CBP
Ligand
E401→Q
E454→Q
Δ403
G303→E

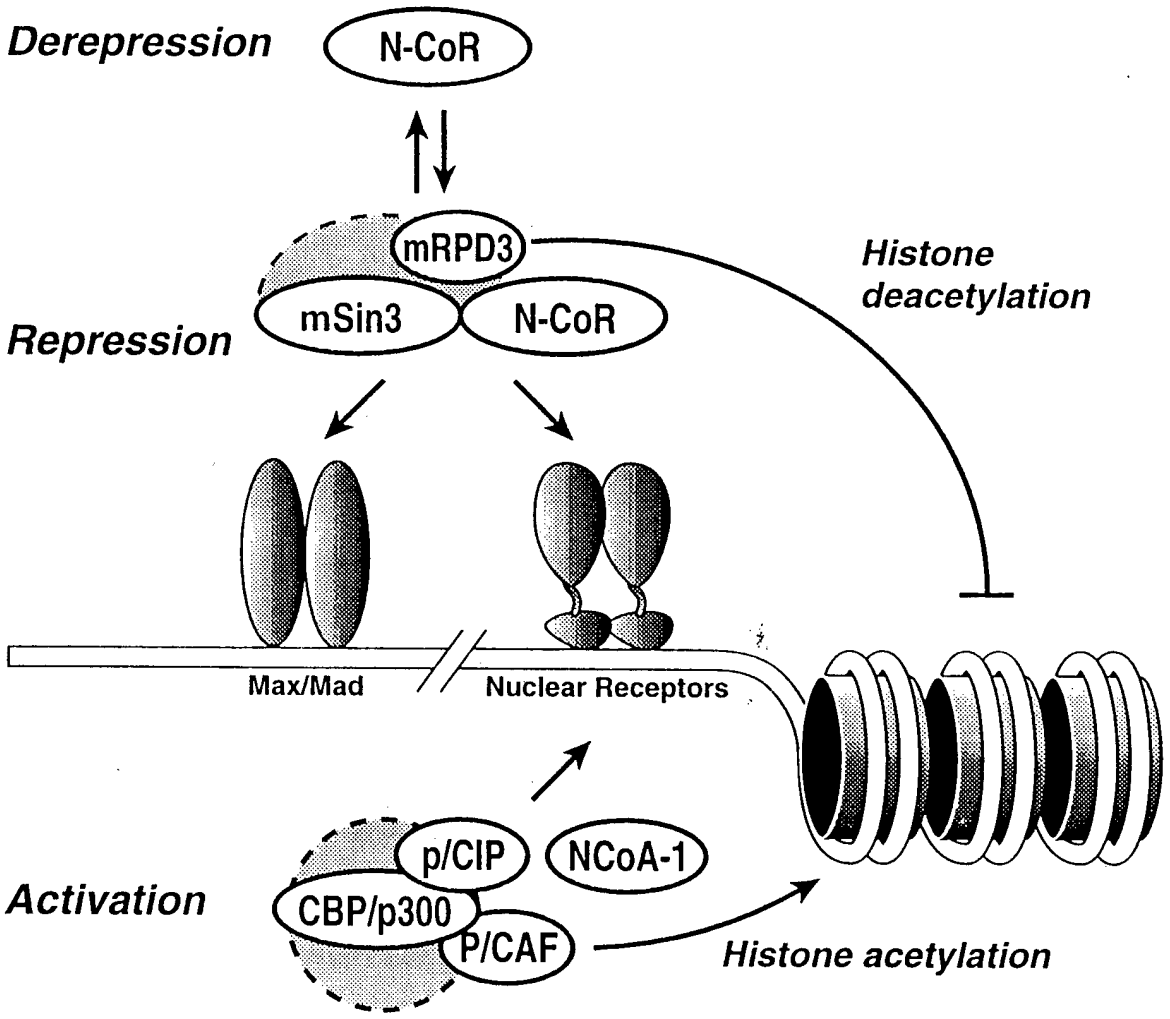

A
TK Luc
RARE Luc
Fold Induction
CBP (μg)
0
0.5
1.0
3.0
B
CBP
HPV-16
AP1/p36
T3
GAL4-T3R-C'
CBP
HPV-16
UAS/tk
C
no RA
RA
anti-CBP (1 mg/ml)
anti-CBP (3 mg/ml)
% blue cells
RARE p36/lacZ
RARE SV40/lacZ
IgG
anti-CBP
% cells expressing β-galactosidase
SV40 lacZ
CMV lacZ



D

```
SRC-1a     1 MSGLGDSSSDPANPDSHKRKGSPCDTLASSTEKRRREQENKYLEGLAELLSANISDIDSLSVKPDKCKILKKTVDQIQLMKRMEQEKSTTDDDVQESDIS
SRC-1a   101 SSSQGVIEKESLGPLLEALDGFFFVVNCEGRIVFVSENVTSYLGYNQEELMNTSVYSILHVGDHAEFVKNLLPKSLVNGVPWPQEATRRNSHTFNCRML
SRC-1a   201 IHPPEDPGTENQEACQRYEVMQCFTVSQPKSIQEDGEDFQSCLICIARRLPRPPAITGVESFMTKQDTTGKIISIDTSSLRAAGRTGWEDLVREGIYAF
SRC-1a   301 FQPQGREPSYARQLFQEVMTRGTASSPSYRFILNDGTMLSAHTKCKLCYPQSPDMQPFIMGIHIIDREHSGLSPQDDSNSGMSIPRINPSVNPGISPAHG
SRC-1b                                                                  MSFSPREHSGLSPQDDTNSGM...
SRC-1a   401 VTRSSTLPPSNNNMVSARVNRQQSSDLNSSSSHTNSSNNQGNFGCSPGNQIVANVALNQGQAGSQTTNPSLNLNNSPMEGTGIALSQFMSPRRQANSGLA
SRC-1a   501 TRARMSNNSFPPNIPTLSSPVGITSGACNNNNRSYSNIPVTSLQGMNEGPNNSVGFSAGSPVLRQMSSQNSPSRLSMQPAKAESKDSKEIASILNEMIQS
SRC-1a   601 DNSDNSANEGKPLDSGLLHNNDRLSEGDSKYSQTSHKLVQLLTTTAEQQLRHADIDTSCKDVLSCTGTSSSASSNPSGGTCPSSHSSLTERHKILHRLLQ
SRC-1a   701 EGSPSDITTLSVEPEFKDSVPASTAVSVSGQSQGSASIKLELDAAKKKESKDHQLLRYLLDKDEKDLRSTPNLCLDDVKVKVEKKEQMDPCNTNPTPMTK
SRC-1a   801 PAPEEVKLESQSQFTADLDQFDQLLPTLEKAAQLPSLCETDRMDGAVTGVSIKAEVLPASLQPTTARAAPRLSRLPELELEAIDNQFGQPGAGDQIPWAN
SRC-1a   901 NTLTTINQNKPEDQCISSQLDELLCPPTTVEGRNDEKALLEQLVSFLSGKDETELAELDRALGIDKLVQGGGLDVLSERFPPQQATPPLMMEDRPTLYSQ
SRC-1a  1001 PYSSPSPTAGLSGPFQGMVRQKPSLGAMPVQVTPPRGTFSPNMGMQPRQTLNRPPAAPNQLRLQLQQRLQGQQQLMHQNRQAILNQFAANAPVGMNMRHG
SRC-1a  1101 MQQQITPQPPLNAQMLAQRQRELYSQQHRQKQIIQQQKPMLMKHQSFGNNIPPSSGLPVQMGDPRLLQGAPQQFPYPPNYGTNPGTPPASTSPFSQLAAN
SRC-1c                                                                                                ...SFSGRGSNSGIS*
SRC-1a  1201 PEASLATRSSMVNRGMAGNMGGQFGAGISPQMQQNVFQYPGPGLVPQGEATFAPSLSPGSSMVPMPVPPPQSSLLQQTPPTSGYQSPDMKAWQQGTMGNN
SRC-1d                              ...TGTAPIPGYWKTFCCI*
SRC-1a  1301 NVFSQAVQSQPAPAQPGVYNNMSITVSMAGGNANIQNMNPMMGQMQMSSLQPGMNTVCSEQMNDPALRHTGLYCNQLSSTDLLKTDADGTLQVQQMVQV
SRC-1e                                                                                                ...NQDKETEEFF
SRC-1a  1401 FADVQCTVNLVGGDPYLNQPGPLGTQKPTSGPQTPQAQQKSLLQQLLTE*
SRC-1e       SVVTTD*
```

Figure 4


Derepression
N-CoR
mRPD3
mSin3
N-CoR
Repression
Histone
deacetylation
Max/Mad
Nuclear Receptors
p/CIP
NCoA-1
CBP/p300
P/CAF
Activation
Histone acetylation